A surface antigen influenza vaccine.
2. Pyrogenicity and antigenicity

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SUMMARY

Conventional influenza vaccine containing whole virus particles purified on a zonal centrifuge is pyrogenic and can cause systemic and local adverse side effects. An improved vaccine was therefore prepared which contained only the surface antigens of the virus adsorbed to aluminium hydroxide. The antigenicity of this vaccine was compared with conventional vaccine in chickens. Both vaccines induced similar titres of serum haemagglutination-inhibition and neuraminidase inhibition antibody. The dose response curves, however, were different. The surface antigens at vaccine strength without aluminium hydroxide were of negligible pyrogenicity in rabbits.

INTRODUCTION

Influenza vaccines containing intact virus particles sometimes cause adverse reactions both systemically and at the site of injection. Over the past few years the purity of these vaccines has steadily improved (Reimer *et al.* 1967) but even zonal purified virus can be too reactogenic for use in sensitive individuals or young children (Peck, 1968; Quilligan, Salgado & Alena, 1961).

Simple disruption of the virus structure, without any further purification, has been shown to reduce the incidence of side-reactions (Duxbury, Hampson & Sievers, 1968; Hennessy & Davenport, 1974). Certainly any factor which interferes with the structural integrity of viral lipids reduces pyrogenicity (Webster & Laver, 1966), even if the lipids themselves remain in the vaccine. However, a vaccine which still contains all the products of disruption will indiscriminately induce antibodies both to the extraneous viral components and to the protective surface antigen.

Various workers have shown that only antibody to the haemagglutinin and neuraminidase surface antigens is associated with protection (Slepushkin *et al.* 1971; Dowdle, Mostow, Coleman & Kaye, 1973; Hobson, Curry, Beare & Ward-Gardner, 1972). It, therefore, seemed logical to purify these two proteins and hence selectively elicit protective antibody instead of inducing a mixture of antibodies.

A technique has been described previously (Brady & Furminger, 1976) for disruption of influenza virus and separation of the surface antigens from the rest of the virus components. The surface antigens were adsorbed to aluminium hydroxide, which acted as a carrier for the purified proteins to increase their effective size and enhance antigenicity.

The reactions and potency observed with this type of vaccine are discussed.

MATERIALS AND METHODS

Virus

A/England/42/72 influenza was obtained from the World Influenza Centre, Mill Hill, London, and was cultivated as previously described (Brady & Furminger, 1976).

Vaccine

The virus was purified, disrupted with Triton N 101 and the haemagglutinin and neuraminidase proteins were isolated as previously described (Brady & Furminger, 1976).

Alhydrogel (Superphos Ltd) (2 %, w/v, $Al(OH)_3$ in water) was added to the concentrated subunits after removal of Triton. The suspension was allowed to stand overnight at 4° C. and then diluted to the required strength (400 International Units (i.u.)/ml.). Final vaccine contained $Al(OH)_3$ (5 mg./ml.) in 0·1 M phosphate buffer.

Haemagglutination assay (HA) (W.H.O. 1953)

Serial doubling dilutions of virus (0·25 ml.) were made in World Health Organization (W.H.O.) plastic plates containing 0·01 M pH 7·6 phosphate buffered saline (PBS) (0·25) in each well. Chick red blood cells (0·25 ml. of a 0·5 %, v/v, suspension in PBS) were added and the plates were left for 1 hr. at room temperature. The titre was recorded as the dilution at which 50 % settling of the cells occurred.

Haemagglutination-inhibition assay (HAI)

Serum was incubated with five volumes of cholera filtrate at 37° C. for 18 hr. to destroy non-specific inhibitors of haemagglutination. The sample was then kept at 56° C. for 30 min. to destroy heat labile inhibitors and to inactivate the neuraminidase in cholera filtrate. Serial doubling dilutions were made in PBS on plastic plates and 8 HAU of virus was added to each well in a 0·25 ml. volume. The plates were left at room temperature for 30 min. and then chick red blood cells were added. The plates were left for a further hour at room temperature. A 100 % end-point was recorded as the last dilution of serum where the cells settled completely.

Neuraminidase assay

To standardize vaccines, the neuraminidase assay of Warren (1959) was automated according to the method of Kendal (1967). The incubation mixture consisted of 0·2 ml. fetuin (24 mg./ml. water), 0·2 ml. phosphate buffer (0·4 M, pH 5·9), 0·2 ml. saline (0·85 %, w/v) and 0·2 ml. of sample containing neuraminidase. An arbitrary value of 100 neuraminidase units was assigned to a virus concentrate which gave an absorbance of 0·8 at 550 nm. after sixteen hours' incubation at 20° C. All vaccines were compared with this standard.

Neuraminidase inhibition (NI)

NI antibody was measured by incubating $0.5 \log_{10}$ dilutions of inactivated (56° C. for 30 min.) serum with either a recombinant virus (H Fowl Plague N_2) or purified homologous haemagglutinin and neuraminidase subunits in the presence of Triton. These indicator virus systems were used instead of homologous virus to avoid steric hindrance owing to HAI antibody. The indicator virus was diluted so that it gave an absorbance at 550 nm. of about 0.8, after 16 hr. incubation at 37° C. A 50 % inhibition end-point was recorded.

Radial diffusion

Haemagglutinin was assayed by a single-radial diffusion technique (Schild, Henry-Aymard & Pereira, 1972) using antiserum prepared in rabbits to bromelain derived haemagglutinin (Brand & Skehel 1972).

Pyrogen assay

The pyrogenicity of the vaccine was assessed according to the method described in the European Pharmacopœia (1973).

EXPERIMENTAL

Pyrogenicity of haemagglutinin and neuraminidase proteins

Haemagglutinin and neuraminidase proteins were prepared from zonal purified virus by centrifugation through a gradient containing Triton as previously described (Brady & Furminger, 1976). The Triton was removed by phase-separation after addition of phosphate buffer.

The subunits were diluted with pyrogen-free 0.01 M phosphate buffered saline pH 7.6 (PBS) to 400 International Units (i.u.)/ml. and the preparation was injected into three rabbits at 1 ml. per rabbit and into another three rabbits at 8 ml. per rabbit. The control was zonal purified whole virus at 400 i.u./ml. (1 ml. per rabbit).

Rectal temperatures were recorded at hourly intervals for 6 hr. and the mean change in temperature with time was recorded.

RESULTS

Fig. 1 shows the difference in temperature response to zonal purified whole virus and the surface antigen preparation. The whole virus vaccine at 10,000 HAU/ml. (400 i.u.) caused a maximum increase in temperature of 1.28° C. whereas a similar dose of surface antigen vaccine caused a rise in temperature of 0.03° C. Even increasing the dose of subunit vaccine to 3200 i.u. only produced a temperature rise of 0.3° C.

These results show that by disruption of virus and removal of lipid, it is possible to isolate the surface antigens in non-pyrogenic form.
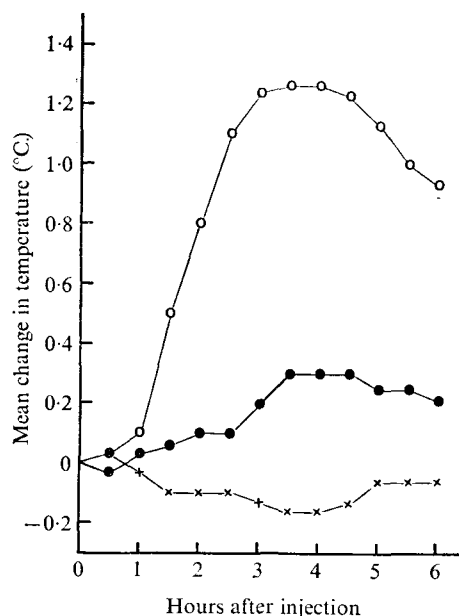
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Fig. 1. Pyrogenicity of zonal purified A/England/42/72 whole virus and the purified haemagglutinin and neuraminidase aqueous surface antigen vaccine (400 i.u./ml.) was injected into three rabbits at 1 ml. per rabbit (×——×) and 8 ml. per rabbit ●——●. The mean increase in temperature was compared with the response induced by 400 i.u. of a zonal purified whole virus vaccine ○——○.

Potency of surface antigen vaccine in chickens

Haemagglutinin and neuraminidase subunits prepared on a sucrose gradient as described previously were adsorbed to aluminium hydroxide and diluted to 400 i.u./ml.

For the potency estimation the adsorbed surface antigen vaccine was diluted 1/3, 1/10 and 1/100 in 0·1 M phosphate buffer containing 5 mg./ml. Al(OH)$_3$. Similar dilutions of aqueous whole virus vaccine (400 i.u./ml.) were made in PBS.

Each vaccine was injected intramuscularly neat and at the three dilutions into five chickens on day 0 and day 14. Blood samples were taken by cardiac puncture before vaccination and on day 14 and day 28 after vaccination. The serum was separated and tested for HAI and NI antibody.

Tables 1 and 2 show the number of chickens with the stated titre at each vaccine dilution. The primary response to the surface antigen vaccine was low or absent but the secondary response compared favourably with the secondary response to the aqueous whole virus vaccine. The HAI responses to the aqueous whole virus vaccine were 4656, 1014, 507 and 2 for the neat, 1/3, 1/10 and 1/100 dilutions, and the HAI titres for similar dilutions of surface antigen vaccine were 543, 666, 1536 and 55.

The dose response relation for the two vaccines was different. A 'plateau-effect' was obtained for the adsorbed surface antigen vaccine whereas for aqueous vaccine the decline in titre was proportional to dilution.

Table 1. *Distribution of A/England/42/72 serum HAI antibody responses in chickens to aqueous whole virus vaccine and adsorbed surface antigen vaccine*

		Reciprocal HAI titre											GMT
		< 12	12	24	48	96	192	384	768	1536	3072	6144	
		Aqueous whole virus vaccine											
1/1	a*	.	.	.	.	1	.	1	.	2	.	1	882
	b*	.	.	.	.	.	.	.	.	.	2	3	4656
1/3	a	1	.	2	2	.	.	.	.	.	.	.	17
	b	.	.	.	.	.	.	1	1	3	.	.	1014
1/10	a	3	1	.	1	.	.	.	.	.	.	.	4
	b	.	.	.	1	.	.	.	3	1	.	.	507
1/100	a	5	.	.	.	.	.	.	.	.	.	.	0
	b	4	.	.	.	1	.	.	.	.	.	.	2
		Adsorbed surface antigen vaccine											
1/1	a	2	1	2	.	.	.	.	.	.	.	.	6
	b	.	.	.	.	1	.	.	2	1	.	.	543
	a	3	.	.	1	1	.	.	.	.	.	.	5
1/3	b	.	.	.	.	1	.	.	1	2	.	1	666
1/10	a	2	2	1	.	.	.	.	.	.	.	.	5
	b	.	.	.	.	.	1	.	1	.	2	1	1536
1/100	a	5	.	.	.	.	.	.	.	.	.	.	0
	b	1	.	.	2	1	1	.	.	.	.	.	55

* *a* and *b* are sera taken 2 and 4 weeks after vaccination respectively.
Five chickens were used for each dilution. Deaths occurred where the total is less than 5.

Table 2. *Distribution of A/England 42/72 serum NI antibody responses in chickens to aqueous whole virus vaccine and adsorbed surface antigen vaccine*

		Reciprocal NI titre										GMT
		< 10	10	15	20	30	40	50	60	80	120	
		Aqueous whole virus vaccine										
1/1	a*	4	.	1	.	.	.	.	.	.	.	2
	b*	1	1	.	.	1	.	.	1	.	1	18
1/3	a	5	.	.	.	.	.	.	.	.	.	0
	b	3	1	.	1	.	.	.	.	.	.	3
1/10	a	5	.	.	.	.	.	.	.	.	.	0
	b	5	.	.	.	.	.	.	.	.	.	0
1/100	a	5	.	.	.	.	.	.	.	.	.	0
	b	5	.	.	.	.	.	.	.	.	.	0
		Adsorbed surface antigen vaccine										
1/1	a	5	.	.	.	.	.	.	.	.	.	0
	b	.	.	.	3	1	.	.	.	.	.	22
1/3	a	5	.	.	.	.	.	.	.	.	.	0
	b	.	.	.	.	.	2	.	.	1	.	50
1/10	a	5	.	.	.	.	.	.	.	.	.	0
	b	2	1	.	.	.	1	.	.	.	1	9
1/100	a	5	.	.	.	.	.	.	.	.	.	0
	b	4	.	.	.	.	.	.	.	.	.	0

Notes as for Table 1.

DISCUSSION

The surface antigens, haemagglutinin and neuraminidase, isolated from A/England/42/72 virus were shown to be much less pyrogenic in rabbits than whole virus even when 3200 i.u. of the surface antigens were used. The pyrogen test was performed, of necessity, on an aqueous suspension of haemagglutinin and neuraminidase subunits. A reduction of pyrogenicity has been reported previously as a consequence of virus disruption by ether (Davenport et al. 1964), tri-n-butyl phosphate (Ruben & Jackson, 1972) or sodium deoxycholate (Duxbury et al. 1968; Webster & Laver, 1966). The cause is considered (Grossebauer, Langmaäck, Schmidt & Kucchler, 1969; Siegert & Braune, 1964) to be disturbance of the spatial arrangement of the viral lipids and occurs even if the lipids are not subsequently removed.

It is of great advantage to have a vaccine which does not induce a febrile response because it can be used safely in young children, whereas vaccine containing intact virus often gives distressing symptoms (Warburton & Duxbury, 1973).

The disadvantage of disruption is the concomitant loss of antigenicity. To some extent the more efficient the breakdown process and the smaller the subunits, the greater is the loss in potency. Vaccines prepared by ether treatment tend to contain fairly large (80S) rolled up pieces of surface projections and are equivalent in antigenicity to whole virus (Davenport et al. 1964). A split product similar in morphological appearance and potency to ether-disrupted virus was obtained with tri-n-butyl phosphate. However, the potency was strain-dependent and the split products of some strains were of much lower antigenicity than whole virus (Neurath et al. 1970).

Vaccines prepared from sodium deoxycholate split influenza virus contain smaller aggregates of antigen (24S) than ether vaccines. In the absence of existing antibody two doses were required to stimulate protective titres of serum HAI antibody, although in the presence of existing antibody the deoxycholate vaccine gave results comparable with intact virus vaccine (Duxbury et al. 1968). Similar results were found by Webster & Laver (1966) who measured the primary and secondary responses to sodium deoxycholate disrupted vaccine in rabbits.

The loss in antigenicity seems to be greater if the surface antigens are separated from the viral proteins irrelevant to protection. Thus, the purified haemagglutinin obtained after bromelain treatment (Brand & Skehel, 1972) was of low potency in hamsters (Jennings et al. 1974) unless it was used in Freund's adjuvant.

The surface antigens described in this report were prepared and purified on a sucrose gradient containing Triton N101 (Brady & Furminger, 1976) and were adsorbed to a carrier to enhance potency. A pyrogen-free suspension of aluminium hydroxide was used for this purpose and the potency of the adsorbed surface antigens was compared with aqueous whole virus vaccine in chickens.

The potency was measured by the induction of serum HAI and NI antibody. The chickens were given two doses of vaccine to try to overcome the problem of using an unprimed animal to test the efficacy of a vaccine which would be used in human population which has undergone previous exposure to influenza. In

chickens this vaccine exhibited a plateau effect and was more antigenic at lower doses than the aqueous whole virus vaccine. A similar plateau effect has been observed in humans (Brady, Furminger & Stones, 1976).

The vaccine has been shown to protect both ferrets (Brady & Furminger, 1975) and hamsters (Jennings *et al.* 1975) against challenge with the homologous strain of virus. It also protects humans against challenge with an attenuated homologous strain of virus (Potter *et al.* 1976).

In all these systems the purified haemagglutinin and neuraminidase antigen vaccine adsorbed onto aluminium hydroxide produced a serum antibody response and protected the animals against challenge. This vaccine is thus protective and non-pyrogenic and should give rise to fewer adverse reactions than the current whole virus vaccines.

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